Potential of Using Culture Filtrates from *Verticillium albo-atrum* to Evaluate Alfalfa Germ Plasm for Resistance to Verticillium Wilt

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ABSTRACT

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The potential was assessed for using cellfree filtrates from potato-dextrose broth cultures of *Verticillium albo-atrum* in a fast, effective screening procedure for Verticillium wilt resistance in alfalfa. In one assay, cut stems were allowed to imbibe filtrate and were rated for symptoms 7 days later. The symptoms that developed in susceptible cuttings after this treatment were typical of those in field-infected alfalfa. Six percent of the cuttings from susceptible Saranac were rated resistant by this procedure, compared with 32 and 56% from resistant Maris Kabul and NAPB-34, respectively. Control cuttings treated with uninoculated potato-dextrose broth or autoclaved water did not develop symptoms. Similar results were obtained with susceptible cuttings after uptake of filtrate from *V. albo-atrum* cultured in alfalfa decoction medium. In the second assay, filtrate was perfused by pressure into the leaflets. Susceptible leaflets became chlorotic after 4–6 days. Seventeen percent of Saranac and 70% of NAPB-34 plants were rated resistant by this method. Control treatments of uninoculated culture medium or autoclaved water did not cause symptoms. The activity of the filtrate remained stable after autoclaving or freezing.

Additional key words: disease resistance, lucerne, *Medicago sativa*

Verticillium wilt, caused by *Verticillium albo-atrum* Reinke & Berth., is a major disease of alfalfa (*Medicago sativa* L.) in the northern United States (1). Host resistance is the most practical means for controlling this disease. Although effective, the currently used root-inoculation screening procedure (15) is laborious and time consuming. It permits too many escapes, is restricted to those geographic locations where the disease exists, and has slow symptom expression. A rapid, dependable screening procedure that minimizes escapes would facilitate both commercial and research breeding programs.

Various fungal and bacterial filtrates and/or toxins have been used to screen plants for disease resistance. Filtrates from *V. dahliae* cultures have been tested on tobacco (12) and cotton (9). A significant correlation was obtained between the reactions of alfalfa plants to the toxin from *Corynebacterium insidiosum* and to the bacterium (19). Cell lines of alfalfa showed resistance when grown on media containing filtrates from *Fusarium oxysporum* f. sp. *medicaginis* cultures (6), and plants regenerated from these lines were resistant when inoculated with the fungus. Some *Helminthosporium* species produce toxins that can be used to screen host germ plasm for resistance to the respective pathogen (5,18,20).

There is good evidence that *V. albo-atrum* produces a toxin(s) in alfalfa that is partially responsible for the foliar symptoms observed (14), and the use of cellfree filtrates from *V. albo-atrum* cultures to screen alfalfa for resistance has been proposed. Panton (14) observed chlorosis, necrosis, and vascular browning in alfalfa seedlings after treatment with heated and unheated filtrates from *V. albo-atrum* cultures. In a similar study, Michail and Carr (13) obtained a strong correlation between the *V. albo-atrum* filtrate-induced reactions of seedlings and the disease ratings of older plants. Although these researchers demonstrated that filtrates from *V. albo-atrum* cultures were biologically active, they did not develop systems adapted to large-scale mass-selection breeding programs. The objective of this research was to determine if alfalfa responses to culture filtrates of *V. albo-atrum* could be used as a rapid, reliable assay for Verticillium wilt resistance.

MATERIALS AND METHODS

Alfalfa growth conditions. One hundred plants each of Saranac (susceptible) and Maris Kabul (resistant) alfalfa were grown in a peat-vermiculite substrate in the greenhouse. Light was supplemented with metal halide lamps to a minimum intensity of 210 μE m^{-2} sec^{-1} for a 12-hr photoperiod. Plants were inoculated with *Rhizobium* and fertilized every 6–8 wk. NAPB-34 (resistant) alfalfa was grown from seed in a growth chamber under a 16-hr photoperiod (90 μE m^{-2} sec^{-1} at plant height) at 22 C during the day and 17 C at night. These plants were fertilized every 4–6 wk. The same plants were used in all studies.

Preparation of filtrate. *V. albo-atrum* was isolated from infected alfalfa plants collected in Pennsylvania. Surface-sterilized basal stem pieces were placed on 1.5% water agar (WA) and incubated until conidia were observed on the stem surfaces. Spores from stem pieces were streaked onto WA. Single-spore cultures were established and maintained on potato-dextrose agar (PDA).

Potato-dextrose broth (PDB) was inoculated with plugs of agar containing *V. albo-atrum* and incubated on rotary shakers at 99 rpm and 24 C for 2 wk in the

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dark. The broth cultures were then filter-sterilized by passing them, under vacuum, through a series of filters with decreasing porosity; the last one had a pore diameter of 0.2 μm. The filtrate and PDB were used fresh or after storage at −10 C. Control treatments were autoclaved tap water and PDB shaken and filtered in the same manner as the filtrate.

To provide a culture substrate more closely related to the natural plant, an alfalfa decoction broth was prepared by air-drying stems and leaves of Saranac alfalfa for 2 days and irradiating them with gamma rays (2.5×10^{-6} rads) for 6–8 hr. Two grams of the sterile alfalfa tissue was added to flasks containing sterile distilled water. The alfalfa broth was inoculated, incubated, and stored in the same manner as the PDB.

Stem cutting assay. Stem cuttings from each of 10–15 plants per cultivar (one stem per plant) were placed upright in a beaker containing 100 ml of either 1:1 filtrate/autoclaved tap water, 1:1 PDB/autoclaved tap water, or autoclaved tap water. After 24 hr, all solutions were replaced with 100 ml of tap water.

For studies involving only Saranac, the cuttings were taken from the same population of 40 plants growing in the greenhouse. Treatments were 1:1 filtrate/autoclaved tap water, 1:1 broth/autoclaved tap water, and autoclaved tap water. Ten cuttings (one per plant) were used per treatment, and the cuttings were rated after 7 days.

Treated stems were kept in a growth chamber at 15–17 C and a 16-hr day length (200 μE m^{-2} sec^{-1}). The cuttings were rated after 7 days on a scale of 1–5, where 1 = green leaves; 2 = general chlorosis of leaflets; 3 = leaf necrosis with or without chlorosis and leaf curl on <50% of stem; 4 = leaf necrosis and curl on >50% of stem; and 5 = wilted stem with chlorosis, necrosis, and leaf curl. Cuttings with ratings of 1 or 2 were classified as resistant; those with ratings of 3–5 were susceptible. All experiments were repeated.

Leaflet infiltration assay. Cultivars Saranac and NAPB-34 were assayed by this method. Filtrate, PDB, or sterile distilled water was forced from a 10-ml syringe into the intercellular spaces of a leaflet by a modification (F. L. Lukezic, *personal communication*) of the technique developed by Klement (8). The syringe needle was inserted through a rubber stopper that had a hemispherical cavity in its distal surface. The needle projected only into the cavity so as neither to contact nor puncture the leaflet surface, and the stopper surface peripheral to the cavity sealed the stopper against the leaflet surface. The stopper was held firmly against the upper surface of a leaflet, which was supported underneath by finger pressure, and pressure applied to the syringe forced the liquid into the leaflet. When adequately infiltrated, a leaflet appeared dark and water-soaked.

The leaflets remained attached to the stems, and after treatment, the cuttings were kept in a growth chamber under the same conditions as in the previous assay until symptoms developed (5–7 days). Leaflet response was rated as chlorotic (susceptible) or green (resistant). Data are based on the treatment of 50 leaflets (one per stem) for Saranac and NAPB-34. Subsequent experiments were done with intact plants under the same incubation conditions and also with treated plants kept in a dark chamber with saturated humidity at 20 C.

RESULTS

Stem cutting assay. In susceptible reactions, cuttings displayed Verticillium wilt-like symptoms after uptake of the filtrate (Fig. 1). Leaves first became chlorotic, then developed a pinkish necrosis that usually began at the margins of the leaflets and progressed inward. Leaflets often curled inward or twisted around the midvein as they became necrotic and dried. Sometimes leaflets or entire petioles abscised. The stem remained green and upright, and new green leaves emerged. The earliest symptoms were observed 48 hr after treatment with filtrate, and severe symptoms developed on susceptible cuttings after 5 days.

Leaves on cuttings from Saranac and Maris Kabul cultivars became chlorotic, and some had severe symptoms 7 days after uptake of filtrate. Saranac had the highest mean symptom severity score, 4.0, and the lowest frequency of resistant plants, 6% (Table 1). The mean score for Maris Kabul was significantly lower at 2.9, and 32% of the cuttings were rated resistant.

NAPB-34 cuttings did not become chlorotic in filtrates, but some displayed a white necrosis on the margins of the leaflets. These were given a rating of 3 or

Fig. 1. Appearance of cuttings from (left) resistant and (right) susceptible alfalfa 7 days after treatment with culture filtrates of *Verticillium albo-atrum*.

Table 1. Symptom severity scores and percentage of plants rated resistant with culture-filtrate stem-cutting assay and the percentage of plants rated resistant by inoculation with *Verticillium albo-atrum* (data from other tests) for Saranac, Maris Kabul, and NAPB-34 alfalfa

Cultivar origin of cuttings	Symptom severity score[a]	Percent plants rated resistant by	
		Culture-filtrate assay[b]	Inoculation (data source)
Saranac (S)[c]	4.0	6	10 (17)
Maris Kabul (R)	2.9	32	30–67 (3,10)
NAPB-34	2.0	53	77 (J. B. Moutray)
LSD	0.36	0.16	

[a] Based on a five-point severity scale where 1 = lowest and 5 = highest.
[b] Mean of about 50 cuttings per cultivar, challenged with a 1:1 dilution of filtrate.
[c] S = susceptible and R = resistant to Verticillium wilt.

4 depending on severity. NAPB-34 had a mean severity score of 2.0, which was significantly lower than those of the other two cultivars, and it had a significantly higher frequency of resistant plants, 53% (Table 1).

No cuttings from any cultivar treated with PDB or water controls showed severe symptoms by day 7. In these treatments, the mean severity scores were all under 2.5. By 7 days, some leaflets on cuttings receiving the water or PDB were chlorotic, but necrosis was rare.

Saranac cuttings immersed in fresh filtrate, filtrate that had been autoclaved for 20 min at 121 C, or filtrate that had been frozen (−10 C) for 3 mo had mean symptom severity scores of 4.0, 4.0, and 4.1, respectively. The activity of filtrate from a 6-wk-old PDB culture of *V. albo-atrum* did not differ significantly from that of filtrates from 2-wk-old cultures. The activity of *V. albo-atrum* filtrate on Saranac did not lessen during nine monthly transfers on PDA.

Saranac cuttings had a mean severity score of 4 when fed a 1:1 dilution of filtrate from inoculated alfalfa decoction broth and of 2.5 with uninoculated alfalfa broth. These differences were significant ($P = 0.01$) but not different from results obtained using the PDB culture filtrates.

Leaflet infiltration assay. Chlorosis and necrosis were induced within 4–6 days in leaflets of susceptible plants perfused with filtrates from *V. albo-atrum* (Fig. 2). Chlorosis developed in 83% of Saranac leaflets but in only 30% of NAPB-34 leaflets perfused with filtrate. None of the control leaflets from either cultivar became chlorotic when perfused with water or dilutions of PDB. Symptoms developed only on the treated leaflet and not on other leaflets on the same petiole or other leaves on the same stem. Treated leaflets on intact plants reacted in the same way as those on detached stems. Posttreatment incubation in a dark moist chamber lengthened the time for water-soaking to disappear but did not alter symptom response.

DISCUSSION

Culture filtrates have potential for use in screening alfalfa for resistance to *V. albo-atrum*. Both the stem cutting and leaflet infiltration assays could reduce the time required for a selection cycle compared with the currently used root-soak method (15).

An effective screening procedure has to be amenable to testing a large number of plants, and it should be simple, relatively rapid, and significantly differential (4). That is, it should quickly and easily differentiate between susceptible and resistant genotypes. Both of the assays evaluated satisfied these criteria and could be used in breeding programs to screen large numbers of plants rapidly or to check symptomless plants for possible escapes in a conventional screening program.

Susceptible alfalfa cuttings treated with filtrate developed symptoms typical for *V. albo-atrum*-infected alfalfa in the field (2,7). This supports the premise that the culture filtrate is challenging the same host systems as those challenged by fungal inoculation. Other host plants showed symptoms similar to those occurring from infection (5,11,18) when treated with fungal filtrates and toxins. The cuttings in PDB and water did not become as desiccated as those in filtrate. Thus, fungal metabolites were probably responsible for the symptoms observed. Saranac cuttings had the same severity score when treated with filtrates from *V. albo-atrum* cultured on alfalfa stem and leaf decoction medium as in filtrates from *V. albo-atrum* grown in PDB. This suggests that alfalfa is nutritionally able to support the production of compounds by *V. albo-atrum* that have the same effect on susceptible cuttings as those produced in PDB.

The incidence of resistant plants identified with the stem cutting assay correlated well with published resistance ratings (3,10,17; J. B. Moutray, *personal communication*). Ratings of the number of resistant plants in any specific cultivar vary considerably even when the same inoculation method is used. The assignment of resistant and susceptible categories to specific response ratings also contributes to the differences observed between evaluations.

The leaflet infiltration assay offers some advantages over both the root-soak (15) and stem cutting assays. Infiltration evokes a localized response involving only a few treated leaflets per plant. It could be done on intact plants wherever they are growing, and because the leaflets take on a water-soaked appearance when adequately infiltrated, the chance of escapes is extremely low with this procedure.

Filtrates and toxins from pathogens remain stable over time and when subjected to temperatures higher than 100 C (14,18), and the apparent *V. albo-atrum* toxin in culture filtrates behaves similarly. The temperature stability of the filtrate allows it to be stored for long periods of time or to be shipped. This could facilitate screening for resistance to *V. albo-atrum* in areas where the pathogen is not present. Filtrates could also be used to identify susceptible germ plasm for research purposes without infecting the plants.

V. albo-atrum disrupts normal plant functions in several ways, including disrupting xylem vessel differentiation and impeding water movement through the host (16). Different genotypes could have different mechanisms of resistance to *V. albo-atrum*, and it is possible that the filtrate would not adequately screen for all types of resistance, possibly only for resistance to the toxin.

Problems could arise when trying to standardize filtrate activity. In this study, attempts were made to standardize the amount of inoculum per culture medium and the environmental conditions for filtrate production. Although this does not ensure complete uniformity of the filtrate activity, susceptible alfalfa consistently gave similar reactions to filtrates produced at different times. Resistant and susceptible checks should be included with each assay.

Other problems could arise if undiluted filtrate were too active to give a good differential response between resistant and susceptible germ plasms. Standardizing filtrate activity through the responses of clones to various dilutions would increase reliability and reproducibility within a filtrate screening system.

Fig. 2. Range of responses of leaflets from (upper row) NAPB-34, resistant, and (lower row) Saranac, susceptible, 5 days after perfusion with culture filtrates of *Verticillium albo-atrum*.

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